The Journal of Molecular Diagnostics, Vol. 17, No. 5, September 2015

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the Journal of Molecular Diagnostics
jmd.amjpathol.org

Droplet Digital PCR for Absolute Quantification of *EML4-ALK* Gene Rearrangement in Lung Adenocarcinoma

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Accepted for publication April 14, 2015.

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Crizotinib treatment significantly prolongs progression-free survival, increases response rates, and improves the quality of life in patients with *ALK*-positive non—small-cell lung cancer. Droplet Digital PCR (ddPCR), a recently developed technique with high sensitivity and specificity, was used in this study to evaluate the association between the abundance of *ALK* rearrangements and crizotinib effectiveness. FFPE tissues were obtained from 103 consecutive patients with lung adenocarcinoma. Fluorescent *in situ* hybridization (FISH) and ddPCR were performed. The results revealed that 14 (13.6%) of the 103 patients were positive by dual-color, break-apart FISH. Three variants (1, 2, and 3) of the *EML4-ALK* gene rearrangements were detected. Thirteen of 14 *ALK*-positive cases identified by FISH were confirmed by ddPCR (four with variant 1, two with variant 2, and seven with variant 3). The case missed by ddPCR was identified as *KIF5B-ALK* gene rearrangement by PCR-based direct sequencing. Sixteen patients were detected with low copy numbers of *EML4-ALK* gene rearrangement, which failed to meet the positive cutoff point of FISH. Two of them responded well to crizotinib after unsuccessful chemotherapy. Our study indicates that ddPCR can be used as a molecular analytical tool to accurately measure the *EML4-ALK* rearrangement copy numbers in FFPE samples of lung adenocarcinoma patients. *(J Mol Diagn 2015, 17: 515—520; http://dx.doi.org/10.1016/j.jmoldx.2015.04.002)*

Lung cancer is the leading cause of cancer-related death worldwide. Recently, successful strategies to improve personalized lung cancer therapy have focused on the epidermal growth factor receptor (*EGFR*)—targeted therapies, such as tyrosine kinase inhibitors (TKIs) (gefitinib or erlotinib).[1] A recent study suggested that a gene rearrangement comprising portions of the *EML4* gene and the *ALK* gene in non—small-cell lung cancer (NSCLC) cells might be a promising therapeutic target and a diagnostic molecular marker.[2] When compared with chemotherapy, crizotinib (a targeted inhibitor of ALK and C-met) used in second-line treatment significantly prolonged progression-free survival (PFS), increased response rates, and improved the quality of life in patients with advanced and *ALK*-positive NSCLC.[3]

ALK-rearranged lung cancer can be identified by fluorescent *in situ* hybridization (FISH), immunohistochemistry (IHC), and RT-PCR. FISH is recommended by the National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology for NSCLC (version 3, 2014) for the clinical diagnosis of *ALK*-positive lung cancer. However, FISH requires specialized technical resources and expertise.[4] FISH diagnosis is very subjective, which limits accurate quantification. IHC is relatively inexpensive and can be routinely performed on formalin-fixed, paraffin-embedded (FFPE) tissues. However, IHC is a rapid screening method but associated with relatively low sensitivity and specificity, except when the ALK monoclonal antibody D5F3 and the Ventana Diagnostic System (Ventana Medical Systems, Illkirch Graffenstaden, France) were used.[5] RT-PCR is commonly used to identify

Supported by grant 81472189 from the National Natural Science Foundation of China (Y.H.) and grant 2012AA02A201 from 863 Major Project of China (Y.H.).

Disclosures: None declared.

http://dx.doi.org/10.1016/j.jmoldx.2015.04.002

Table 1 Clinical Profile of Genetically Screened Patients with Lung Adenocarcinoma

Characteristic	No. (%) of patients, $N = 103$	No. (%) *ALK* (FISH) positive
Sex		
Male	51 (49.5)	8 (15.7)
Female	52 (50.5)	6 (11.5)
Smoking status		
Never-smokers	66 (64.1)	9 (13.6)
Smokers	37 (35.9)	5 (13.5)
Specimen type		
Surgical	57 (55.3)	9 (15.8)
Biopsy	46 (44.7)	5 (10.9)
Total	103	14 (13.6)

FISH, fluorescence *in situ* hybridization.

known fusion variants of *ALK* gene rearrangements with high sensitivity but semiquantitatively.[6] Treatment with EGFR TKIs was effective in advanced NSCLC because of the abundance of *EGFR* mutations.[7] The median PFS and OS rates of patients with low abundance of *EGFR* mutations were shorter than those with high abundance but significantly longer than those with wild-type tumors. It is still unclear if the abundance of *ALK* gene rearrangements was predictive of treatment efficacy with crizotinib. Unfortunately, the current methods are not adequate to detect the abundance of *ALK* gene rearrangements accurately.

Droplet Digital PCR (ddPCR), a recently developed technique, involves emulsification and PCR amplification inside thousands of small droplets, each droplet containing one or no molecules of target DNA or RNA.[8] Precise and absolute quantification of the number of target DNA or RNA molecules in the reaction is simply achieved by counting the number of positive and negative droplets.[9] The strategy reduces competitive amplification, allowing detection of 0.001% mutant fractions, which is 1000 times lower than real-time PCR. In this study, we used ddPCR to reveal a highly sensitive and specific detection of *EML4-ALK* gene rearrangements in mRNA derived from clinical lung adenocarcinoma FFPE samples. We further discuss the potential benefit of crizotinib in the treatment of patients with low copy numbers of *ALK* rearrangements.

Materials and Methods

Patients and Tissues

This study was approved by the Clinical Ethics Committee of Daping Hospital and Research Institute of Surgery, Third Military Medical University. Signed informed consent was obtained from all patients. FFPE tissues were obtained from 103 consecutive patients with lung adenocarcinoma patients between January 2013 and February 2014. They were randomly selected after diagnosis of lung adenocarcinoma, and none of them had received ALK inhibitor treatment or chemotherapy before surgery or biopsy. The clinical data and smoking history of the patients were obtained from their medical records.

RNA Isolation and cDNA Synthesis

Total RNA was extracted and purified from freshly cut FFPE tissue sections using the RNeasy kit (Qiagen, Duesseldorf, Germany), after ascertaining the tumor percentage in each specimen. The RNA quality was verified by NanoDrop 2000 (Thermo Fisher Scientific, Carlsbad, CA), and not more than 1 μg of RNA was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA). The total volume of cDNA obtained from the reverse transcription reaction was 20 μL.

FISH

FISH was performed on unstained 4-μm-thick FFPE specimens using the Vysis LSI ALK dual-color, break-apart FISH probes (Abbott Molecular, Des Plaines, IL), according to the manufacturer's instructions. Slides were read on a fluorescence microscope (BX51; Olympus, Tokyo, Japan) and independently evaluated by two experienced pathologists (Q.M. and H.X.). Tumor cells containing at least one pair of green and orange signals, split apart by ≥2 signal diameters, or a single orange signal without the corresponding green signal were diagnosed as positive for *ALK* gene rearrangement. The criteria of 15% break-apart signals or orange single signals isolated in 50 tumor cells were used as the *ALK*-positive cutoff levels.[10]

ddPCR Workflow

The ddPCR was performed as previously described.[11,12] TaqMan reaction mixture was assembled from a 2× ddPCR master mix (BioRad Laboratories, Hercules, CA), 20× primers and probes (final concentrations of 900 and 250 nmol/L, respectively), and 1 μL of cDNA in a final volume of 20 μL. Each reaction mixture was loaded into a sample well of an eight-channel disposable droplet generator cartridge (BioRad Laboratories). The emulsified samples were generated from a droplet generator (QX100; BioRad Laboratories) and then transferred onto a 96-well plate. After heat-sealing with a foil seal, the emulsified samples underwent a 2-step thermal cycling protocol in a C-1000 touch thermal cycler (BioRad Laboratories) as follows: 95°C ×10 minutes, 40 cycles of 95°C ×30 seconds and 60°C ×60 seconds (ramp rate set to 2°C per second), and 98°C ×10 minutes. The 96-well droplet PCR plates were loaded into a droplet reader (BioRad Laboratories), which automatically reads the droplets from each well of the plate. Analysis of the ddPCR data were performed with QuantaSoft analysis software version 1.6 (BioRad Laboratories). Three main variants of *EML4-ALK* were analyzed in this study, and forward primers of these variants were as follows: 5′-AGCCCACACCTGGGAAAGGAC-3′ for variant 1, 5′-CAGCTACATCACACACCTTGACTGGT-3′